The Heparin-binding Exosite of Factor IXa is a Critical Regulator of Plasma Thrombin Generation and Venous Thrombosis

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ABSTRACT:

The role of the factor IXa heparin-binding exosite in coagulation was assessed with mutations that selectively enhance (R170A) or reduce (R233A) stability of the protease-factor VIIIa A2 domain interaction. Following tissue factor (TF) addition to reconstituted factor IX-deficient plasma, factor IX R170A supported a 2-fold increase in velocity index (slope) and peak thrombin concentration, while factor IX R233A had a 4-10 fold reduction relative to factor IX wild type. In the absence of TF, 5-100 pM factor IXa increased thrombin generation to approach TF-stimulated thrombin generation at 100% factor IX. Factor IXa R170A demonstrated a 2-3 fold increase in peak thrombin concentration and up to 5-fold increase in velocity index, while the response for factor IXa R233A was blunted and delayed relative to wild type protease. In hemophilia B mice, factor IX replacement reduced the average time to hemostasis (ATTH) following saphenous vein incision, and the time to occlusion (TTO) following FeCl3-induced saphenous vein injury. At 5% factor IX, the TTO for factor IX wild type, R170A, and R233A were 15.7, 9.1 (P ≤ 0.003), and > 45 min. These data support the role of the factor IXa heparin-binding exosite as a critical regulator of coagulation and novel antithrombotic target.
**Introduction:**

Thrombin is the penultimate product of the coagulation cascade, generated in an explosive burst upon stimulation of plasma with limiting concentrations of tissue factor\(^1,2\). The measurement of plasma thrombin generation has merits as a global test of coagulation, and enhanced levels of thrombin generation have been associated with increased risk of recurrent venous thrombosis \(^3\). Thus, the rate and magnitude of thrombin generation may be predictive of the coagulation phenotype of individuals\(^4,5\). In *vitro* and *ex vivo* modeling of the coagulation cascade indicates that factor X activation by the intrinsic tenase complex (factor IXa-factor VIIIa) is the rate-limiting step for thrombin generation \(^1,2,6\). Intrinsic tenase activity is unstable due to the diffusional loss of the noncovalently-bound factor VIIIa A2 domain \(^7,8\). The instability of this enzyme complex is presumed to be an important regulator of the coagulation response.

The mechanism(s) for activation of factor IXa within the intrinsic tenase complex are poorly understood. Factor VIII circulates as a heterodimer of A1-A2-B and A3-C1-C2 peptides with domain structure and metal-binding functions similar to ceruloplasmin \(^9\). Factor VIII undergoes proteolytic activation by thrombin, resulting in an unstable, metal-dependent A1/A2/A3-C1-C2 heterotrimer with cofactor activity\(^10,11\). Factor VIII or factor VIIIa light chain (A3-C1-C2 domains) bind to factor IXa on the phospholipid surface with an affinity that approaches intact factor VIIIa, but lack cofactor activity\(^12,13\). The isolated factor VIIIa A1 domain also lacks cofactor activity. In contrast, the factor VIIIa A2 domain directly modulates the catalytic activity of factor IXa, which is further enhanced by the A1 domain, markedly increasing the \(k_{cat}\) for factor X activation \(^14,15\). Although the isolated A2 domain binds with low affinity to factor IXa, it contributes significantly to protease-cofactor affinity in the membrane-
bound enzyme complex \textsuperscript{16}. Thus, the factor IXa-A2 domain interaction appears to be the critical protein-protein interface for cofactor enhancement of factor X activation.

The heparin-binding exosite on the factor IXa protease domain is a cofactor interactive site that contributes significantly to stabilization of the factor VIIIa A2 domain, and allosteric activation of the protease within the enzyme complex \textsuperscript{17,18}. This exosite is the molecular target for antithrombin-independent inhibition of the intrinsic tenase complex by heparin and other glycosaminoglycans, and may contribute to the antithrombotic properties of heparin \textsuperscript{17,19}. Mutations in the factor IXa heparin-binding exosite that modulate intrinsic tenase stability can be used to assess the importance of the factor IXa-A2 domain interaction in complex systems. Factor IXa R170A (chymotrypsinogen numbering system) demonstrates increased apparent cofactor affinity and enhanced stability of the enzyme complex with purified components, and increased coagulant activity in an APTT-based assay. In contrast, factor IXa R233A demonstrates decreased apparent cofactor affinity and reduced stability of the enzyme complex with purified components, and moderately reduced coagulant activity \textsuperscript{17}. Coagulant activity measured in APTT-based assays is useful for the detection of factor deficiencies, but represents a highly unphysiologic assessment of blood coagulation. The complexity of \textit{ex vivo} plasma and \textit{in vivo} injury models make predictions regarding the importance of specific molecular interactions difficult. However, the opposing phenotypes of these recombinant proteases provide valuable tools to investigate the role of the factor IXa-A2 domain interaction in more physiologic systems.

Since evidence suggests that interaction with the factor VIIIa A2 domain is critical to cofactor activation of factor IXa, factor X activation by the intrinsic tenase complex is rate-limiting for thrombin generation, and thrombin generation phenotype is associated with the risk of venous thrombosis, we hypothesized that the factor IXa heparin-binding exosite is a critical regulator of coagulation response.
To test this hypothesis, we evaluated the effect of mutations in the heparin-binding exosite of factor IX (R170A and R233A) on tissue factor and factor IXa-initiated thrombin generation in factor IX-deficient plasma, bleeding from a saphenous vein incision, and FeCl₃-induced saphenous vein thrombosis in the hemophilia B mouse. The results suggest that the heparin-binding exosite of factor IXa is a critical physiologic regulator of plasma thrombin generation and venous thrombus formation.

**Materials and Methods:**

*Materials:* Human normal pooled plasma and factor IX-deficient patient plasma were purchased from George King (Overland Park, KS). Corn trypsin inhibitor (CTI) was obtained from Haematologic Technologies (Essex Junction, VT). Human plasma-derived factor IX, IXa, and thrombin were purchased from Enzyme Research (South Bend, IN). Phosphatidylserine (PS) and phosphatidylcholine (PC) were purchased from Avanti Lipids (Alabaster, AL). Cholesterol was purchased from Calbiochem (San Diego, CA). Phosphatidylcholine: phosphatidylserine:cholesterol (molar ratio 75:25:1) phospholipid vesicles (PC:PS vesicles) were prepared by extrusion through a 100 nm polycarbonate filter. DMSO was purchased from Mallinckrodt (St. Louis, MO), bovine serum albumin (A-9647) from Sigma (St. Louis, MO), and urea from EM Sciences (Cherry Hill, New Jersey). Lyophilized bovine thrombin-α₂-macroglobulin complex was purchased from Thrombinoscope BV (Maastricht, The Netherlands). Thromborel S, a human thromboplastin from Dade Behring (Deerfield, IL), was used as the source of relipidated human tissue factor. The tissue factor concentration was 200 ng/ml based on the mean of ELISA results for multiple lots of Thromborel S (Stephanie Smith, University of Illinois, email communication 3/02/06). The fluorogenic substrate Z-Gly-Gly-Arg-AMC HCl was obtained from Bachem (King of Prussia, PA).
Expression and purification of recombinant factor IX: HEK 293 cell lines stably transfected with human factor IX wild type and R170A were provided by Darrel Stafford (University of North Carolina-Chapel Hill) 21. A stable HEK 293 cell line expressing human factor IX R233A was constructed as described 17. Recombinant factor IX proteins were purified to homogeneity from conditioned media, and quantitated by absorbance at 280 nm. A portion of the factor IX was activated to factor IXa with human factor Xla, and factor IXa catalytic sites quantitated by titration with antithrombin III, as previously described 18.

Fluorogenic method for detection of plasma thrombin generation- Thrombin generation in human plasma was detected by modification of the methods of Hemker et al. and Technothrombin® TGA 3,22. The assay was performed in 96-well polystyrene flat-bottom clear plates from Greiner (Monroe, North Carolina). Thrombin activity was determined by AMC (7-amino-4-methylcoumarin) release from the fluorogenic substrate Z-Gly-Gly-Arg-AMC, detected by a 360/40-nm-excitation and 460/40-nm-emission filter set in a Biotek Synergy HT fluorescent plate reader equipped with Gen 5 software (Biotek Instruments, Inc., Winooski, VT). The substrate Z-Gly-Gly-Arg-AMC HCl was reconstituted at 100 mM in DMSO and stored at -20°C. Prior to each assay, a fresh fluorogenic substrate and calcium solution (FluCa substrate) was prepared by adding 100 µl of 1.0 M CaCl₂ to 875 µl of 20 mM HEPES, pH 7.35, 60 g/l BSA at 37°C, followed by 25 ul of 100 mM Z-Gly-Gly-Arg-AMC in DMSO with vigorous mixing. FluCa substrate had a final concentration of 2.5 mM Z-Gly-Gly-Arg-AMC HCl and 100 mM CaCl₂. The bovine thrombin-α2-macroglobulin complex calibrator was reconstituted in distilled water, followed by dilution in TGA buffer (100 mM NaCl, 20 mM HEPES, pH 7.35, and 1% BSA) to achieve final plasma concentrations of 500, 250, 50 and 5 nM, respectively. Factor IX-deficient plasma (60 µl) and TGA buffer containing calibrator (20 µl) were added to each well and preheated at 37°C for 10 min. FluCa substrate (20 µl) at 37°C was then added, mixed at medium
intensity for 5 sec, and readings were obtained at 30 sec intervals for 60 min. The raw data for the first 10 min was imported into Technothrombin TGA evaluation software from Technoclone (Vienna, Austria) to construct the calibration curve.

Plasma thrombin generation was triggered by addition of a limiting tissue factor concentration (0.2 pM) or factor IXa to recalcified plasma. Plasma was thawed sequentially on ice for 5 min, at room temperature for 5 min, and in a 37°C water bath for 5 min. For tissue factor triggered assays, the initiator solution was composed of 0.12 mg/ml corn trypsin inhibitor (CTI), 25 µM PC:PS vesicles, 0.6 pM tissue factor, and 0-270 nM plasma-derived or recombinant FIX in TGA buffer. For factor IXa triggered assays, tissue factor was omitted, and plasma-derived or recombinant factor IXa replaced factor IX. Plasma (60 µl) and initiator solution (20 µl) were added to each well and preheated at 37°C for 10 min. FluCa substrate (20 µl) at 37°C was then added, mixed at medium intensity for 5 sec, and readings were obtained at 30 sec intervals for 60 min. Final concentrations (extrapolated to the 60 µl plasma volume) were 0.2 pM tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI. Fluorescent signal data were exported to Technothrombin TGA evaluation software, and thrombin generation over time was determined using the calibration curve. Numerical parameters generated by the software to describe each thrombin generation curve, included lag time (start until first burst in thrombin formation), peak thrombin concentration, time to thrombin peak, and velocity index (slope between the end of lag time and peak thrombin).

The time course of plasma thrombin generation by Western Blotting- Tissue factor-dependent thrombin generation assays in factor IX deficient plasma supplemented with 4.5 nM recombinant factor IX (5%
plasma levels) were prepared as described above in 1.5 ml eppendorf tubes at 37°C. Individual reactions were quenched by addition of 100 µl 2X SDS-PAGE loading buffer containing 5 M urea. Quenched samples were incubated at 37°C for 5 min, followed by boiling for 5 min, to assure complete clot dissolution. Samples were diluted 1:20 in either non-reducing or reducing SDS-PAGE loading buffer and subjected to 10% SDS-PAGE, followed by overnight transfer to Immobilon-P (Millipore, Billerica, MA). Membranes were blocked in 5% milk and 1% BSA for 60 min, incubated in a 1:2500 dilution of polyclonal sheep anti-human thrombin antibody (Haematologic Technologies Inc., Essex Junction, VT) for 90 min, followed by incubation in a 1:10000 dilution of peroxidase-conjugated affinity purified donkey anti-sheep IgG (Jackson Immunoresearch, West Grove, PA) for 60 min. The washed Immobilon-P membrane was immersed in Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min and exposed to Kodak BioMax Xar film (Kodak, Rochester, New York).

Wild type and Hemophilia B mice- All procedures were reviewed and approved by the University of North Carolina at Chapel Hill School of Medicine IACUC. Wild-type C57BL/6 (wtC57B6) mice were purchased from Charles River Laboratories (Wilmington, MA) and/or bred in house. Hemophilia B knockout mice were the kind gift of Dr. Dougal M. Monroe.

Saphenous vein hemostasis and thrombosis models- Adult mice were anesthetized with 0.1 ml/g body weight of a 2.5% 2,2,2-tribromoethanol (Sigma-Aldrich, St. Louis, MO) solution. Ventral hind limb hair was removed bilaterally and the animal placed supine on a THM 100 Temperature & ECG Monitoring Board (Indus, Instruments, Houston, TX). The paws were gently restrained by Velcro attached the ECG pads and/or by a loop of PE tubing attached to the board. The skin overlying both the left and
right ventral hind limb was incised, a length of the saphenous neurovascular bundle exposed and covered with normal saline to prevent drying of the tissues. To allow for injection of therapeutic agents the left saphenous vein was cannulated under a SZX12 dissecting microscope (Olympus, Melville, NY) using a catheter constructed of pulled PE-10 tubing (Braintree Scientific, Inc., Braintree, MA) with a 3.0 mil cleaning wire (Hamilton Co., Reno NV) placed into the lumen as a stylet. For the factor IX dose response curves, mice were given weight-dependent doses of plasma-derived factor IX (Mononine, ZLB Behring, Kankakee, IL) in 0.9% sodium chloride to achieve 0-100% normal plasma concentrations (0-1 U/mL). For recombinant human factor IX wild type, R170A, or R233A experiments, mice were given weight dependent doses of a 200 nM stock solution recombinant human factor IX to achieve 10% normal plasma concentration (0.1 U/mL) for the hemostasis model, or 5% normal plasma concentration (0.05 U/mL) for the thrombosis model. All factor IX injections were given 5 min prior to the initiation of saphenous vein incision or FeCl₃ induced injury. All animals were euthanized at the end of the procedure.

Hemostasis was assessed by determination of serial bleeding times from a saphenous vein incision. The right saphenous vein was transected by piercing through and through with a 23g needle (Becton Dickinson & Co, Franklin Lakes, NJ). Blood was wicked away with a Kimwipe (Kimberly-Clark, Roswell, GA) until hemostasis occurred (typically 1-2 min for all mice). A longitudinal incision was made in the distal portion of the vessel using Student Vannas Spring Scissors (Fine Science Tools, Foster City, CA) by inserting one blade into the vessel as far as possible and then cutting. Blood was gently wicked away with a Kimwipe until hemostasis occurred. The clot was then disrupted using a 30g needle, and blood wicked away until hemostasis occurred again. Clot disruption was repeated after
every incidence of hemostasis until 30 minutes after the initial 23 g needle injury. All injury, clot
disruption, and hemostasis time points were recorded on the Chart™ software.

Thrombosis in the saphenous vein was produced using FeCl$_3$ (Sigma Chemical Co., St. Louis, MO)
induced injury. Flow in the right saphenous vein was monitored using a 20 MHz Doppler flow probe
(Indus, Instruments, Houston, TX). Data from the Doppler probe was collected on an Apple iMac using
a PowerLab 4/20 and Chart™ software (ADInstruments Pty Ltd., Colorado Springs, CO). Five minutes
after recombinant factor IX injection, vessel injury was initiated by placing a 0.5 X 2 mm strip of filter
paper soaked in 10% FeCl$_3$ onto the vein. After 2 minutes, the filter paper was removed, the
neurovascular bundle washed twice with normal saline and then covered again with normal saline and
the flow signal reacquired. Injury and thrombosis were allowed to proceed until vessel occlusion. The
time to occlusion (TTO) was determined from application of the filter paper until loss of the Doppler
flow signal for one minute. The experiment was stopped after vessel occlusion, or 30 or 45 minutes
(factor IX dose response curve or recombinant human factor IX experiments, respectively) after filter
paper was applied if no occlusion occurred.

Results:

Effect of mutations in the factor IX heparin binding exosite on tissue factor-stimulated plasma thrombin
generation. To assess the effect of these mutations on the ability of recombinant factor IX to support
plasma thrombin generation triggered by tissue factor, factor IX-deficient plasma was supplemented
with 0, 1, 5, 10, and 100% (90 nM) levels of each recombinant factor IX. A dose-dependent increase in
velocity index (slope) and peak thrombin concentration, modest reduction in lag time, and shortening of
the time to peak thrombin generation was observed between 0-100% factor IX levels in all mutants (Fig 1A-C). Thrombin generation curves of factor IX R170A were shifted modestly to the left compared to factor IX wild type (Fig 1A, B). This mutant was able to generate significant amounts of thrombin even at 1% factor IX levels (Fig 1B), and demonstrated 2-fold enhancement in velocity index and peak thrombin concentration between 1-5% levels compared to factor IX wild type (Fig 1D, E). In contrast, factor IX R233A thrombin generation curves were shifted substantially to the right (Fig 1C) with significantly reduced velocity index and peak thrombin concentration (4-10 fold) compared to factor IX wild type (Fig 1D, E). At factor IX R233A levels of 10% or less, thrombin generation was markedly blunted (Fig 1C), and even at 100% plasma levels still generated less thrombin than 5% levels of factor IX wild type (Fig 1A, C).

**Western Blot of plasma thrombin generation in the presence of 5% recombinant factor IX**- To confirm that fluorogenic substrate cleavage accurately represented plasma thrombin generation by recombinant factor IX mutants, the time course of thrombin activation and inhibition products was monitored by Western blot. The primary anti-thrombin antibody simultaneously detected prothrombin, thrombin-related cleavage products, and the thrombin-antithrombin complex (TAT). Thrombin generation was initiated by 0.2 pM human tissue factor in factor IX-deficient plasma supplemented with 5% recombinant factor IX, and reaction products were analyzed by SDS-PAGE under non-reducing (Fig 2) and reducing (data not shown) conditions. Thrombin generation in the presence of 5% factor IX wild type demonstrated appearance of the TAT complex at 4 min with progressive accumulation over time, and peak free thrombin concentration at 10 min (Fig 2A). An unidentified higher molecular weight band also accumulated with time, which disappeared under reducing conditions (not shown). Modest reduction in the prothrombin/meizothrombin band was noted over time, without evidence of prothrombin depletion. In the presence of 5% factor IX R170A, the TAT complex was detected at 4.5
min, peak free thrombin accumulation occurred at 7.5 min, and the amount of free thrombin generated was enhanced relative to factor IX wild type (Fig 2B). In the presence of 5% factor IX R233A, thrombin generation was markedly delayed and reduced in intensity, with small amounts of TAT complex appearing at 10 min, and no discernible amount of free thrombin detected up to 16 min (Fig 2C).

*Tissue factor-independent thrombin generation by recombinant factor IXa in factor IX-deficient plasma*

To exclude the potential effects of these mutations in the heparin binding exosite on factor IX activation by tissue factor-factor VIIa complex, thrombin generation was initiated with recombinant factor IXa in the absence of tissue factor. Significant thrombin generation was observed with as little as 5 pM wild type recombinant factor IXa, representing activation of less than 0.001% of the normal zymogen concentration (Fig 3A). A dose-dependent increase in the velocity index and peak thrombin concentration was observed between 5 and 100 pM factor IXa wild type (Fig 3A). Increased concentrations of factor IXa R170A demonstrated a progressive increase in velocity index and peak thrombin concentration, and shortened the time to peak thrombin (Fig 3B). The dose response for factor IXa R170A was shifted significantly to the left relative to factor IX wild type, with enhanced thrombin generation between 1 and 100 pM (Fig 3A, B). The peak thrombin concentration was increased 2-3 fold, and the velocity index up to 5-fold for factor IXa R170A relative to wild type protease (Fig 3D, E). Addition of 1 pM factor IXa R170A resulted in similar velocity index and peak thrombin to 100 pM factor IXa wild type (Fig 3D, E), while 1 pM wild type protease failed to trigger a significant thrombin response. Factor IXa R233A demonstrated reduced thrombin generation relative to wild type protease between 5 and 100 pM (Fig 3C). Addition of 1 pM factor IXa R233A to plasma resulted in no significant thrombin response, while the response to 5-10 pM protease was blunted and delayed compared to wild type factor IXa (Fig 3A, C). However, at 100 pM factor IXa, peak thrombin generation by factor IXa R233A approached that of the wild type protease (Fig 3A, C).
**Effect of recombinant factor IX on the average time to hemostasis (ATTH) following saphenous vein incision in hemophilia B mice**- The effect of mutations in the heparin-binding exosite of factor IX(a) on hemostasis in the hemophilia B mouse was examined by bleeding times after a distal saphenous vein incision obtained with repeated clot disruption. The time to hemostasis for each bleeding episode was recorded for 30 min, and the average time to hemostasis (ATTH) for all episodes was calculated for each mouse. Wild type C57BL/6 mice (n = 4) demonstrated a median ATTH of 51 sec, while factor IX-gene ablated C57BL/6 mice (n = 5) had a median ATTH of >1800 sec (3 of 5 mice failed to achieve hemostasis within 30 min) (Fig 4). Recombinant factor IX was infused at weight-based doses sufficient to achieve approximately 10% normal plasma levels prior to saphenous vein incision in hemophilia B mice. Median ATTH for factor IX wild type (n = 4) was 111 sec and factor IXa R170A (n = 4) was 34 sec, both significantly shorter than untreated hemophilia B mice (P ≤ 0.008, Mann-Whitney test). In contrast, factor IX R233A (n = 4) demonstrated a median ATTH of 1304 sec (2 out of 4 mice failed to achieve hemostasis), not significantly different from untreated hemophilia B mice.

**Effect of factor IX on the time to vessel occlusion following FeCl₃ injury of the saphenous vein in wild type and hemophilia B mice**- To determine the factor IX dependence of venous thrombosis formation in response to FeCl₃-induced saphenous vein injury, the time to vessel occlusion as monitored by a Doppler flow probe was determined in both wild type and hemophilia B (factor IX-gene ablated) C57BL/6 mice. Following FeCl₃-induced saphenous vein injury, wild type C57BL/6 mice (n= 4) demonstrated a median time to occlusion (TTO) of 11.5 min, while hemophilia B mice (n= 4) failed to occlude the vessel within 45 min (data not shown). Infusion of plasma-derived factor IX prior to saphenous vein injury shortened the TTO in hemophilia B mice back to values observed in wild type
mice. The dose response for factor IX demonstrated marked shortening of the TTO between 3 and 5% normal plasma factor IX levels, and limited further effect between 5 and 100% levels (data not shown).

**Effect of factor IX mutations on TTO in the hemophilia B mouse at 5% plasma levels**- To determine the effect of mutations in the heparin-binding exosite of factor IX(a) on saphenous vein thrombosis in the hemophilia B mouse, recombinant factor IX was infused at doses sufficient to achieve approximately 5% normal plasma levels prior to vessel injury. Recombinant factor IX wild type demonstrated a median TTO of 15.7 min, which was modestly prolonged relative to the plasma-derived factor IX preparation (Fig 5). Infusion of factor IX R170A at 5% normal plasma levels resulted in a TTO of 9.1 min, which was significantly shortened relative to the wild type protease (P ≤ 0.003, Mann-Whitney test). In contrast, infusion of factor IX R233A at 5% plasma levels resulted in a median TTO greater than 45 min. To confirm that factor IX R233A has in vivo activity, the dose was increased to achieve approximately 50% normal plasma levels of factor IX, which resulted in a TTO similar to the wild type protease (data not shown).

**Discussion:**

We hypothesized that mutagenesis of the factor IXa heparin-binding exosite would dramatically affect the rate of plasma thrombin generation and formation of venous thrombi. Plasma thrombin generation was detected by fluorogenic substrate cleavage and Western blotting for thrombin-related products. Factor IX-deficient plasma supplemented with 100% plasma levels of factor IX demonstrated enhanced thrombin generation compared to normal, pooled human plasma (data not shown). This result may reflect individual characteristics of the patient plasma, or pre-analytic variables related to collection and processing of the pooled human plasma. A single lot of hemophilia B patient plasma was used for
all additional experiments, eliminating pre-analytical variables as the source of variation in thrombin generation. Increasing amounts of wild type factor IX (1-100%) accelerated the time course and amplitude of thrombin generation. Minimal differences in thrombin generation were observed between 0 and 1% wild type factor IX levels, while 5% levels resulted in significantly enhanced thrombin responses, achieving greater than half of the peak thrombin concentration observed with 100% factor IX levels (Fig 1A). These data suggest a threshold effect for enhancement of thrombin generation by factor IX that correlates with the observed clinical severity of mild (> 5%), moderate (1-5%), and severe (< 1%) hemophilia phenotypes 26. Likewise, thrombin generation was exquisitely sensitive to factor IXa concentrations in the 5-100 pM range (Fig 3). Notably, 100 pM factor IXa (0.11% normal zymogen concentration) resulted in thrombin generation similar in magnitude to factor IX-deficient plasma supplemented with 100% factor IX (Figs 1A & 2A). The sensitivity of plasma thrombin generation to picomolar factor IXa is consistent with results obtained from numerical simulations, and in synthetic plasma in the presence of platelets 27. These findings predict that excess factor IX activation will have dramatic effects on thrombin generation and the risk of thrombosis, consistent with the role of factor XI in mouse models of thrombosis 28,29.

The stability of intrinsic tenase activity at subnanomolar concentrations of factor IXa depends largely on diffusional loss of the factor VIIIa A2 domain 7. Recombinant factor IX(a) with mutations in the heparin-binding exosite that selectively enhance (R170A) or reduce (R233A) stability of the protease-A2 domain interaction had dramatic effects on plasma thrombin generation relative to factor IX wild type (Fig 1D, E) 17. Factor IX R170A demonstrated increased velocity-index and peak thrombin concentration relative to wild type protein at all concentrations (Fig 1D), with the relative differences most pronounced at the lowest zymogen concentration (1% plasma levels). In contrast, factor IX R233A demonstrated markedly reduced peak thrombin concentration and velocity index relative to wild type
protein (Fig 1D,E), again most pronounced at the lower zymogen concentrations where no significant thrombin response was observed. This thrombin generation phenotype was more dramatic than suggested by the moderately reduced coagulant activity (59%) of factor IX R233A in an APTT-based assay. Evaluation of plasma thrombin generation by Western blot analysis in factor IX-deficient plasma supplemented with 5% factor IX wild type, R170A, or R233A confirmed the validity of the fluorogenic substrate measurements (Fig 2A-C). When thrombin generation was initiated with factor IXa, the dose response for factor IXa R170A was again shifted to the left relative to wild type, with differences most pronounced at the lower protease concentrations. The dose response for factor IXa R233A was likewise shifted to the right, but approached factor IXa wild type at the highest protease concentration (Fig 3A-C). Thus, the thrombin generation phenotype of these recombinant proteins largely reflects the effect of mutations on protease function. However, sufficiently high factor IXa concentration may compensate for destabilization of the protease-cofactor interaction. The dramatic effect of these mutations on plasma thrombin generation emphasizes the critical role of the interaction between the factor IXa heparin-binding exosite and the factor VIIIa A2 domain in regulation of the coagulation response.

The physiologic relevance of these findings was underscored by the effect of recombinant factor IX on saphenous vein hemostasis and thrombus formation in the hemophilia B mouse. Comparison of wild type and factor IX gene-ablated C57BL/6 mice demonstrated that both bleeding and thrombus formation were factor IX-dependent, and restored in hemophilia B mice by infusion of factor IX prior to the injury. Infusion of 10% plasma levels of recombinant factor IX wild type or R170A into hemophilia B mice dramatically shortened the average time to hemostasis (ATTH) after time saphenous incision, while 10% factor IX R233A was not significantly different from untreated mice (Fig 4). Similar to thrombin generation results, the factor IX dose dependence of thrombus formation after FeCl₃ induced injury
demonstrated a threshold effect below 5% plasma factor IX levels (data not shown). Thus, the magnitude of bleeding risk in hemophilia, plasma thrombin generation, and thrombus formation appear closely linked with respect to factor IX levels. Infusion of a recombinant factor IX dose calculated to achieve 5% plasma concentrations prior to the injury resulted in a significantly shorter TTO (time to occlusion) for factor IX R170A compared to wild type protease, while factor IX R233A failed to trigger thrombus formation at this concentration (Fig 5). These results demonstrate that mutations in the heparin-binding exosite of factor IX have significant effects on in vivo hemostasis and thrombus formation, consistent with their effects on plasma thrombin generation. Thus, stability of the intrinsic tenase complex is critical to both the rate of plasma thrombin generation and formation of venous thrombi in response to injury.

The dramatic effects on intrinsic tenase stability, plasma thrombin generation, hemostasis, and in vivo thrombus formation observed for factor IX R170A and R233A confirm that this heparin-binding exosite is a major physiologic regulator of coagulation. The relative stability of the factor IXa-A2 domain interaction appears to act as a “rheostat” that modulates the magnitude of the coagulation response. These results suggest that manipulation of this protein-protein interaction may have substantial therapeutic value in both hemophilia and thrombosis. Indeed, this protease exosite may contribute to the antithrombotic effects of heparin oligosaccharides and depolymerized holothurian glycosaminoglycan. As opposed to active site-directed inhibitors, targeting this protease exosite may allow down-regulation rather than complete disruption of the coagulation response, resulting in a broader therapeutic window. The ability of these mutations to modulate the factor IXa-A2 domain interaction suggests that this protease exosite is a robust target for novel antithrombotic agents, and amendable to approaches designed to decrease the stability of this critical protein-protein interaction.
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Authorship

Contribution: B.Y. performed the thrombin generation assays and Western blotting, analyzed and interpreted data, and wrote the manuscript. H.C.W. designed and performed the murine hemostasis and thrombosis models, analyzed and interpreted data. J.P.S. designed the research, analyzed and interpreted data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References:


Figure Legends:

Figure 1- Tissue factor-stimulated thrombin generation in factor IX-deficient plasma supplemented with 1, 5, 10, and 100% levels of recombinant factor IX wild type, R170A, and R233A. Thrombin generation was initiated with 0.2 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX deficient plasma supplemented with 0, 1 (0.9 nM), 5 (4.5 nM), 10 (9 nM), and 100% (90 nM) levels of recombinant factor IX wild type (A), R170A (B), or R233A (C). The time course of thrombin generation was measured as described in Material and Methods. Thrombin generation curves representing the mean fluorescent data from replicate determinations (n=10) are shown. Curves are identified by representative points. Peak thrombin concentration (D) and velocity index of thrombin generation (E) for each recombinant protein are plotted for comparison. Error bars show standard error.

Figure 2- Analysis of tissue factor-stimulated thrombin generation by Western blot in factor IX-deficient plasma supplemented with 5% recombinant factor IX wild type, R170A, and R233A. Thrombin generation was initiated with 0.2 pM human tissue factor, 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX deficient plasma supplemented with 5% recombinant factor IX wild type (A), R170A (B), and R233A (C). Individual reactions were quenched over time with loading buffer containing 5 M urea, and analyzed by 10% SDS-PAGE under non-reducing conditions as described in Materials and Methods. Proteins transferred to Immobilon-P were detected with a polyclonal sheep anti-human thrombin primary antibody, followed by a peroxidase-conjugated affinity purified donkey anti-sheep IgG, and subsequent development of signal with chemiluminescent substrate. Prothrombin indicates prothrombin/meizothrombin band. TAT indicates thrombin-antithrombin complex.
Figure 3- Factor IXa-stimulated thrombin generation in factor IX-deficient plasma in the absence of tissue factor. Thrombin generation was initiated by 1-100 pM recombinant factor IXa wild type (A), R170A (B), or R233A (C), in the presence of 8.3 µM PC:PS vesicles, and 40 µg/ml CTI (plasma concentrations) in factor IX deficient plasma. Factor IXa was added just prior to recalcification at a final plasma concentration indicated in the figure. The time course of thrombin generation was measured as described in Material and Methods. Thrombin generation curves representing the mean fluorescent data from replicate determinations (n = 10) are shown. Curves are identified by representative points. Peak thrombin concentration (D) and velocity index of thrombin generation (E) for each recombinant protein are plotted for comparison. Error bars show standard error.

Figure 4- Effect of recombinant factor IX mutants at 10% plasma levels on the average time to hemostasis (ATTH) following saphenous vein incision in the hemophilia B mouse. Wild type or factor IX gene-ablated C57BL/6 mice were subjected to incision of the distal saphenous vein and repeat bleeding times were determined with serial clot disruptions for 30 min, as described in Materials and Methods. The median ATTH (solid lines) for wild type and factor IX gene-ablated C57B6 mice was 51 sec and > 1800 sec, respectively. Factor IX gene-ablated C57BL/6 mice were injected with recombinant factor IX wild type, R170A, or R233A at doses calculated to result in 10% normal plasma levels before saphenous vein incision. Injection of factor IX into the gene-ablated mice shortened the median ATTH for factor IX wild type and R170A to 111 sec and 34 sec, respectively (P≤0.008, Mann Whitney test). Median ATTH for factor IX R233A was 1304 sec (not significant). The y-axis for ATTH represents a log scale.

Figure 5- Effect of recombinant factor IX mutants at 5% plasma levels on the time to occlusion (TTO) following FeCl3-induced saphenous vein injury in the hemophilia B mouse. Factor IX gene-
ablated C57B6 mice were infused with recombinant factor IX wild type, R170A, or R233A at doses calculated to result in 5% normal plasma levels prior to undergoing FeCl₃ induced saphenous vein injury as described in Materials and Methods. Saphenous vein flow was monitored by Doppler probe up to 45 min, or until loss of flow was observed for 60 sec to determine the time to occlusion (TTO). Median TTO (solid lines) was 15.7 min for factor IX wild type, 9.1 min for factor IX R170A, and > 45 min for factor IX R233A (P ≤ 0.003, Mann-Whitney).
Figures

Figure 1- A-E
Figure 2- A-C

A

B

C

kDa 0 2 4 6 8 10 12 14 16 (min)

112 TAT

60 Prothrombin

31 Thrombin

kDa 0 1.5 3 4.5 6 7.5 9 10.5 12 13.5 (min)

112 TAT

60 Prothrombin

31 Thrombin

kDa 0 2 4 6 8 10 12 14 16 (min)

112 TAT

60 Prothrombin

31 Thrombin
Figure 3-A-E
Figure 4-

![Graph showing average time to hemostasis for different factor IX and mouse conditions. The x-axis represents factor IX with categories None, WT, R170A, R233A, and None. The y-axis represents average time to hemostasis in seconds. The graph includes data points and error bars for each condition, comparing FIX-gene-ablated C57BL/6 and C57BL/6 mice.]
Figure 5-

![Graph showing time to occlusion for different recombinant human factor IX concentrations.](image-url)
The heparin-binding exosite of factor IXa is a critical regulator of plasma thrombin generation and venous thrombosis

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